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(71) Applicant: GRANADA BIOSCIENCES, INC. [US/US]; 10900 Richmond Avenue, Houston, TX 77242 (US).

(72) Inventors: BARNES, Frank, L. ; 3010 Aztec, College Station, TX 77845 (US). WESTHUSIN, Mark, E. ; 4024 Viceroy, Bryan, TX 77802 (US).

(74) Agent: BOULWARE, Margaret, A.; One Riverway, Suite 1100, Houston, TX 77056-1903 (US).

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(54) Title: IN VITRO MATURATION OF BOVINE OOCYTES

(57) Abstract

The method of this invention produces mature oocytes from culturing immature oocytes in a maturation media and co-culture which have developmental competence and can be used for nuclear transfer. The maturation media can contain recombinant gonadotropins. The co-culture is media with bovine oviductal cells.

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IN VITRO MATURATION OF BOVINE OOCYTES

Background of the Invention

In vitro maturation of mammalian oocytes has been studied for many years. The ova have been removed from the follicles of the mammals and observed to resume meiosis spontaneously *in vitro*. Edwards, "Maturation In Vitro of Mouse, Sheep, Cow, Pig, Rhesus Monkey and Human Ovarian Oocytes", *Nature*, Vol. 208, pp.349-351 (1965); Leibfried and First, "Characterization of Bovine Follicular Oocytes and Their Ability to Mature In Vitro", *J. Animal Sci.*, Vol. 48, pp.76-86 (1979).

With techniques being developed for *in vitro* fertilization as well as the more recent nuclear transfer techniques in bovine embryos, a source of readily available oocytes would compliment the procedures available for genetic selection and breeding. Immature ova have been collected from bovine ovaries at abbatoirs, and the follicles were aspirated to release cumulus-oocyte complexes. The oocytes have been tested for maturation in media containing hormones and granulosa cells. The interaction of granulosa cells with cumulus complexes have been determined to contribute to developmental competence of bovine oocytes. See *e. g.* Leibfried-Rutledge *et al*, "Development Potential of Bovine Oocytes Matured In Vitro and In Vivo", *Biol. Reprod.*, Vol. 36, pp.376-384 (1987); Critser *et al*, "Acquisition of Developmental Competence During Maturation In Vitro", *Theriogenology*, Vol. 25, No. 1, p.150 (1986); Critser *et al*, "Influence of Cumulus Cell

Association During In Vitro Maturation of Bovine Oocytes on Embryonic Development", Biol. Reprod., Vol. 34 (Suppl.1), p.192 (1986).

Investigators have reported varying results using in vitro fertilization to test the developmental competence of the oocytes. Successful in vitro maturation systems for bovine oocytes yielding fully developed embryos use granulosa cell co-cultures and estrous cow serum. However, the efficiency of this procedure is variable. Lu et al, "Pregnancy Established in Cattle by Transfer of Embryos Derived from In Vitro Fertilization of Oocytes Matured In Vitro", Vet. Rec., Vol. 121, pp.259-260 (1987); Fukui and Ono, "In Vitro Development to Blastocyst of In Vitro Matured and Fertilized Bovine Oocytes", Vet. Rec., Vol. 122, p.282 (1988).

The maturation of both the nuclear material and cytoplasm appears to be important. Activation is the term used for the initiation of the developmental program which normally occurs at the time of fertilization. Activation can be induced by electropulsation without fertilization of the oocyte. Control over activation is important in nuclear transfer to optimize the time to implant the transferred nuclear material which incorporates into and "reconditions" in the presence of recipient maturing ooplasms. The subsequent development is intended to reflect the genetic make-up of the transferred nuclear material after insertion into an enucleated egg. A suitable supply of immature bovine oocytes exists and is inexpensive. A reliable in vitro maturation procedure would give synchronous and measurable oocyte development. Therefore, a mature supply of oocytes would be available for use for genetic selective breeding or cloning.

In vitro matured oocytes for nuclear transfer in cattle has been reported, although embryonic development was limited. Prather et al, "Nuclear Transplantation in

the Bovine Embryo: Assessment of Donor Nuclei and Recipient Oocytes", Biol. Reprod., Vol. 37, pp.859-866 (1987). The oocytes were aspirated from 1-5 mm follicles. The culture medium contained ovine luteinizing hormone (NIADDK oLH-24, 0.012 units/ml), ovine follicle-stimulating hormone (NIADDK oFSH-15, 0.01 units/ml), and estradiol-17B (1 ug/ml). The maturation time was 21 to 27 hours. The hormones used are naturally occurring purified product which contains some comtaminants. However, maintenance of pregnancy from an *in vitro* matured ooctye was not reported by Prather *et al*.

Summary of the Invention

The present invention is a new method for *in vitro* maturation of bovine oocytes. The process can be used to produce oocytes for nuclear transfer, and pregnancies have been confirmed.

Bovine ovaries are collected at abattoirs. Compact cumulus oocyte complexes (COC) were selected preferably from the aspirate of 3-8 mm antral follicles. The COC were placed in a maturation medium containing the gonadotropins luteinizing hormone (LH) and follicle-stimulating hormone (FSH), both purified and recombinant LH and FSH were used. The medium also contained estradiol.

In vitro fertilization was performed with oocytes matured with (1) recombinant FSH and LH, and (2) only the recombinant FSH to determine the activity of the recombinant hormones. These oocytes were not used in nuclear transfer. For the oocytes used in nuclear transfer and other procedures, the cumulus cells were stripped after 20 to 24 hours. The oocytes were incubated in a co-culture of medium plus bovine oviductal cells. The oocytes used for nuclear transfer were aged

from 26 to 43 hours after initial placement in the maturation medium.

The procedure developed for bovine oocyte in vitro maturation with the co-culture feature is successful for use in the nuclear transfer process to produce cloned animals. The use of unique recombinant gonadotropins is another feature of the new process to mature the bovine oocytes.

Detailed Description of the Invention

The following is a description of the process and methods of the invention including the best mode. There may be modifications made to some of the steps used to practice the invention that are apparent to those skilled in the art.

The bovine ovaries were collected at abattoirs and maintained and transported in Dulbeccos PBS at 32° to 39°C. The collection and transport time ranged from 1.5 to 3.75 hours past death. The compact cumulus oocyte complexes (COC) were selected from the aspirate of antral follicles. Some follicles were dissected as discussed below.

The follicle size was investigated as to the best source of oocytes. The most useful range is 3-8 mm. The preferred follicle size determined to give the best oocytes for further developmental competence were 6-8mm. Although the 1-2mm follicles yielded some oocyctes that would mature to develop to morula and blastocyst stage after in vitro fertilization, the development was at a lower frequency. The follicle size was examined in dissected ovaries because 2mm and 3-5mm follicles are difficult to distinguish when measured at the ovarian surface.

The COC were washed three times in Tyrodes-Hepes medium (Bavister, et al, "Development of Preimplantation Embryos of the Golden Hamster in a Defined Culture

Medium", Biol. Reprod., Vol. 28, pp.235-247 (1983)) which is incorporated by reference herein, containing 3mg/ml bovine serum albumin and antibiotic 1% penicillin streptomycin solution (Gibco). After washing, the COC are placed in the preferred maturation media of Tissue Culture Medium (TCM) 199 supplemented with 10% heat inactivated fetal calf serum (HTFCS) and 0.01 units/ml bFSH (recombinant FSH) resuspended in BSA and mannitol solution which is equivalent to 0.01 units of NIADDK-oFSH standard. The recombinant FSH was obtained from Integrated Genetics, Inc., Chappel et al, "Bovine FSH Produced by Recombinant DNA Technology", Theriog., Vol. 29, No. 1 (1988). Esch et al, "Cloning And DNA Sequence Analysis of The DNA for The Precursor of The Chain of Bovine Follicle Stimulating Hormone", Proc. Nat'l Academy Sci. USA, Vol. 83, pp.6618-6621 (1986). The NIADDK-oFSH is a purified natural FSH from the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases (NIADDK), Baltimore, Maryland. The maturation media also contained lug/ml estradiol and 1% penicillin streptomycin solution. The COC are placed in the maturation media for at least 20 hours.

A comparison of other alternative maturation media was performed by subjecting the oocytes from the different media to in vitro fertilization and monitoring subsequent development. The maturation media described above was supplemented with 0.01 units/ml bLH (recombinant LH from Integrated Genetics, Inc.) resuspended in BSA and mannitol solution which is equivalent to 0.01 units of NIADDK-oLH standard. The NIADDK-oLH is a purified natural LH from the National Institute of Arthritis, Diabetes and Digestive and Kidney Diseases, Baltimore, Maryland. Alternative commercially available FSH and LH such as pituitary FSH (pFSH) and pituitary LH (pLH) can be used.

The oocytes were matured in TCM 199, lug/ml estradiol and either 0.01 units/ml of bFSH or 0.01 and

0.012 units/ml of bFSH and bLH, respectively, supplemented with 10% HTFCS. The oocytes were cultured at 39°C 5% CO_2 and air. The oocytes were then assayed for developmental competence by in vitro fertilization followed by culture in ovine oviducts for six days. The addition of bLH adversely affected developmental competence of in vitro matured oocytes although there was some development to the blastocysts and morula stage. TABLE 1 is a summary of the in vitro fertilization results with use of the recombinant gonadotropins.

TABLE 1
RECOMBINANT GONADOTROPINS
IN VITRO FERTILIZATION

Media	Fertilization (%)	Development to Morula or Blastocyst and (%)
bFSH	42/50 (84%)	24/75 (32%)
bFSH + bLH	38/51 (74.5%)	21/128 (16.4%)

In addition, the use of recombinant gonadotropins bFSH and bLH in maturation media was compared to the media with the natural gonadotropins oFSH and oLH supplied by NIADDK. Maturation was carried out in a maturation culture media TCM 199 with 10% HTFCS, 1% penicillin and streptomycin, 1 ug/ml estradiol, 0.01 units/ml of NIADDK-oFSH, 0.012 units/ml of NIADDK-oLH in one media preparation and the equivalent amounts of bFSH and bLH in another media preparation. Bovine oocytes were matured as described above and subjected to in vitro fertilization. The fertilized oocytes were cultured in ovine oviducts and a development to morula or blastocysts was comparable.

The following TABLE 2 shows the results of the NIADDK gonadotropins and the recombinant gonadotropins. The recombinant gonadotropins compare favorably to the NIADDK standards.

TABLE 2
COMPARISON OF GONADOTROPINS

Media	Fertilization (%)	Development to Morula or Blastocyst and (%)
NIADDK Gonadotropins	26/33 (78.7%)	46/103 (44.6%)
Recombinant Gonadotropins	22/30 (73.3%)	26/77 (33.7%)

In vitro oocytes were placed in maturation culture containing both oFSH and oLH to study the ability to activate. Activation was defined as the completion of meiosis and the progression of a metaphase II oocyte to a pronuclear egg. The *in vitro* matured oocytes were removed from the maturation culture media over intervals of 26 to 32 hours as shown in TABLE 3. The oocytes were electropulsed under the same conditions as those used for fusion according to "Bovine Nuclear Transplantation", cited below and incorporated by reference. The following data shows that oocytes 30 hours and older have increased activation potential. The results of the activation procedure are shown below in TABLE 3.

TABLE 3
OOCYTE ACTIVATION

Oocyte Age	Metaphase II	Pronuclear	Lysed
26	15/38 (39%)	18/38 (47%)	5/38 (13%)
28	15/39 (38%)	24/39 (61%)	1/39 (2%)
30	2/32 (6%)	26/32 (81%)	4/32 (12%)
32	1/34 (3%)	27/34 (79%)	6/34 (17%)

Additional aging beyond the period required for acquisition of activation competence (30 hours post introduction into maturation medium) was found to

enhance the developmental potential of the <u>in vitro</u> matured oocytes used in nuclear transfer.

The <u>in vitro</u> oocyte maturation process which sustains nuclear transfer and embryonic development includes a further co-culture procedure. The COC were removed from the maturation media after 20 to 24 hours, preferably 22 hours, and stripped of cumulus cells by vortexing (Vortex Genie 2; shake setting #8) for 2 minutes, 15 seconds in 2ml of Tyrodes-Hepes medium in a 15ml conical tube. About 15 to 30 denuded oocytes of medium color with a polar body were selected and placed into 23ul microdrops and co-cultured with bovine oviductal cells. The polar bodies were visualized with a dissecting microscope 60-120X. The oviductal cell co-culture consisted of 3ul of packed oviductal cells in 20ul of co-culture media TCM 199 with 10% HTFCS and 1% penicillin and streptomycin.

The bovine oviductal epithelial cells were collected from oviductal flushings 36 to 48 hours, preferably 36 hours, after injection of HCG. The preferred oviductal cells for co-culture are predominantly elongated, multicellular, ciliated clumps. The oviductal cells were preconditioned in the co-culture media for 22 to 24 hours prior to the addition of the oocytes. The handling time is 1 to 2 hours prior to introducing the selected oocytes with the co-culture. The oocyte-oviductal cell co-cultures were then incubated at 39°C 5% CO_2 for about 3 to about 5 hours.

The co-culture is supplemented with an equal volume of fresh TCM 199 with HTFCS between 3 to 5 hours after initiating of the co-culture. The co-culture with oocytes was incubated up to 19 hours. Some oocytes were withdrawn at earlier intervals.

Nuclear transfer was performed on oocytes that had been incubated in maturation culture with recombinant bFSH and bLH and co-culture. The oocytes were matured in maturation media for 20 to 24 hours, preferably 22

hours, co-cultured for 3 hours prior to micro-manipulation and then cultured an additional 2 hours in TCM 199 with 10% HTFCS prior to fusion.

Other groups of cells remained in the co-culture for 17 to 19 hours before micro-manipulation for nuclear transfer, fusion and embyronic culture. A comparison was made on the cells in co-culture for about 3 hours to about 5 hours.

The micro-manipulation procedure to enucleate the matured oocytes and transfer nuclear material takes about 4 hours. The nuclear transfer procedure was followed according to "Bovine Nuclear Transplantation", International Application No. PCT/US 88/01906, International Publication No. WO88/091816, 15 December, 1988, which published patent application is incorporated by reference herein.

The preferred method of micro-manipulation in addition to the general technique disclosed in "Bovine Nuclear Transplantation" includes a staining procedure to visualize the female chromatin. The preferred technique provides for removing about 1/2 of the ooplasm from an oocyte and placing the removed ooplasm into a foreign zona pellucida creating two egg halves each with a surrounding zonae. One half should include the female chromatin and one-half should not. With light microscopy, it is impossible to discern the enucleated half without chromatin which is the preferred recipient egg half for the donor nuclear material.

In the preferred procedure, the *in vitro* matured oocyte halves are placed in phosphate saline supplemented with 0.4% BSA, 1% antibiotic penicillin/streptomycin (MPBS) and 5ug/ml Hoechst stain (33342, bisbenzimide trihydrochloride) at 37°C for about 30 minutes. The oocyte halves are then placed in fresh MPBS without stain several minutes and viewed under a fluorescent microscope with the appropriate excitation and barrier filters. Each oocyte half is viewed at 200-400X

magnification using white light. The white light is shut off and the oocyte half is exposed to UV light. The chromatin fluoresces a bright blue and should be located as quickly as possible to reduce the exposure time to UV light. The oocyte halves with chromatin are discarded. The oocyte halves without chromatin are recipients of donor nuclear material.

In some cases, only one of each of the egg halves was stained. In the event the half stained contained the chromatin, the other enucleated half was not exposed to the Hoechst stain. However, the enucleated halves exposed to the Hoechst stain were viable and developed normally.

The fused cells were placed in TCM 199 with 10% HTFCS 0 to 10 hours. The embryos were then cultured according to the procedure described in "Bovine Nuclear Transplantation." The results are shown below in TABLE 4.

TABLE 4
NUCLEAR TRANSFER
IN VITRO MATURED OOCYTES

In Vitro Oocyte Age at Manipulation Approximate Hours	Morula & Blastocysts/Embryos Recovered (%)
25 - 26	17/253 (6.7%)
40 - 43	18/153 (11.7%)

Part of the nuclear transfer study compared *in vitro* oocytes to *in vivo* recovered oocytes and their capability to support development of embryos after nuclear transfer. The nuclear material came from the same blastomeres of one donor embryo. The following TABLE 5 shows the results of the use of *in vivo* matured oocytes and *in vitro* matured oocytes.

TABLE 5
COMPARISON IN VIVO AND IN VITRO OOCYTES

Treatment	Morula & Blastocysts/ Embryos Recovered (%)
In vivo oocyte	3/12 (25%)
In vitro oocyte	6/33 (18%)

There were 36 embryos developing to morula or blastocysts stage. All viable embryos were transferred to synchronous recipient cows. Seven pregnancies have been confirmed.

The method of this invention is a successful *in vitro* oocyte maturation process which can be used for nuclear transfer or other genetic manipulation or fertilization techniques. The method offer an alternative to use of *in vivo* oocytes.

WHAT WE CLAIM IS:

1. A method for *in vitro* maturation of bovine oocytes comprising the steps of

(a) collecting bovine ovaries;

(b) isolating the compact cumulus oocyte complexes;

(c) placing the oocytes in a maturation medium with gonadotropins;

(d) stripping the cumulus cells from the oocytes;

(e) preparing a co-culture of oviductal cells and media; and

(f) aging the oocytes in said co-culture.

2. A method for *in vitro* maturation of bovine oocytes of claim 1 after steps (a) through (f) comprising the steps of

(a') removing the oocytes from the co-culture;

(b') enucleating the oocytes; and

(c') performing nuclear transfer on the enucleated oocytes.

3. A method for *in vitro* maturation of bovine oocytes of claim 1 including adding media to the oocyte and co-culture about 3 hours after initiating step (f).

4. A method for *in vitro* maturation of bovine oocytes of claim 1 wherein said maturation medium comprises bovine gonadatropins selected from the group consisting of recombinant bovine FSH, recombinant bovine LH, oFSH, oLH, pLH, pFSH and mixtures thereof.

5. A method for *in vitro* maturation of bovine oocytes of claim 1 wherein said compact cumulus oocyte complexes are isolated from 3-8mm antral follicles of the bovine ovaries.

6. A method for *in vitro* maturation of bovine oocytes of claim 1 wherein said oviductal cells of the co-culture are preconditioned prior to the addition of the oocytes in media of TCM 199, 10% HTFCS and antibiotics.

7. A method for *in vitro* maturation of bovine oocytes of claim 1 wherein the maturation medium comprises TCM 199, 10% HTFCS, bovine gonadatropins, estradiol and antibiotic.

8. A method for *in vitro* maturation of bovine ooctyes of claim 1 wherein the co-culture comprises oviductal cells, TCM 199, 10% HTFCS and antibiotic.

9. A method for *in vitro* maturation of bovine oocytes of claim 1 wherein the oocytes are held in the maturation medium from about 20 to 24 hours.

10. A method for *in vitro* maturation of bovine oocytes of claim 1 wherein the oocytes are aged in the co-culture from about 3 to 19 hours.

11. A method for *in vitro* maturation of bovine oocytes of claim 1 wherein the oviductal cells of step (e) comprise predominantly bovine epithelial, elongated, multicellular, ciliated clumps.

12. A method for *in vitro* maturation of bovine oocytes of claim 2 wherein after step (a'), the following steps are followed in enucleating the oocyte in step (b') comprising the steps of

(a") removing about half of the ooplasm from a bovine oocyte leaving an oocyte with about half the ooplasm surrounded by the zona pellucida;

(b") evacuating a zona pellucida by removing the ooplasm creating an empty, surrogate zona pellucida;

(c") placing the removed ooplasm from step (a") in the surrogate zona pellucida creating a second oocyte half;

(d") repeating steps (a") through (c") to prepare a selected number of oocyte halves from oocytes and surrogate zona pellucidae;

(e") exposing at least one of each of the oocyte halves prepared in steps (a") through (c") to a vital stain for chromatin;

(f") examining the egg half exposed to the vital stain for chromatin;

(g") discarding the egg halves containing chromatin; and

(h") utilizing those egg halves not containing chromatin as nuclear transfer recipients.

AMENDED CLAIMS

[received by the International Bureau on 1 October 1990 (01.10.90), new claims 13-15 added; other claims unchanged (1 page)]

(b") evacuating a zona pellucida by removing the ooplasm creating an empty, surrogate zona pellucida;

(c") placing the removed ooplasm from step (a") in the surrogate zona pellucida creating a second oocyte half;

(d") repeating steps (a") through (c") to prepare a selected number of oocyte halves from oocytes and surrogate zona pellucidae;

(e") exposing at least one of each of the oocyte halves prepared in steps (a") through (c") to a vital stain for chromatin;

(f") examining the egg half exposed to the vital stain for chromatin;

(g") discarding the egg halves containing chromatin; and

(h") utilizing those egg halves not containing chromatin as nuclear transfer recipients.

13. A method for <u>in vitro</u> maturation of bovine oocytes of claim 1 wherein step 1(d) includes selecting denuded oocytes of medium color with visible polar bodies.

14. A method for <u>in vitro</u> maturation of bovine oocytes of claim 1 wherein the oocytes are held in the maturation medium beyond the period required for activation competence.

15. A method for <u>in vitro</u> maturation of bovine oocytes of claim 1 wherein the oocytes are held in the maturation medium for about 30 hours.

INTERNATIONAL SEARCH REPORT

International Application No PCT/US 90/02365

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[5]: C 12 N 5/06

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[5]	C 12 N

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
X	Biology of Reproduction, vol. 37, 1987, R.S. Prather et al.: "Nuclear transplantation in the bovine embryo: Assessment of donor nuclei and recipient oocyte", pages 859-866 see page 860, "Materials and methods" cited in the application --	1-12
Y	Journal of Reproduction and Fertility, vol. 81, no. 1, 1987, F. Gandolfi et al.: "Stimulation of early embryonic development in the sheep by co-culture with oviduct epithelial cells", pages 23-28 see the abstract --	1-12
Y	Theriogenology, vol. 27, no. 1, January 1987, W.H. Eyestone et al.: "Co-culture of early bovine embryos with oviductal epithelium", page 228 see the whole abstract ----------	1-12

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18th July 1990	07. 08. 90
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	R.J. Eernisse